Molecular Immunology 49 (2011) 483–494

Contents lists available at SciVerse ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm

Antibody repertoire development in fetal and neonatal piglets XXI. Usage of most VH genes remains constant during fetal and postnatal development

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ARTICLE INFO

Article history:
Received 4 August 2011
Received in revised form
22 September 2011
Accepted 24 September 2011
Available online 21 October 2011

Keywords:
VH gene usage
Antigen independent
Developmental immunology
Piglet model

ABSTRACT

Usage of variable region gene segments during development of the antibody repertoire in mammals is unresolved in part because of the complexity of the locus in mice and humans and the difficulty of distinguishing intrinsic from extrinsic influences in these species. We present the first vertical studies on VH usage that spans the fetal and neonatal period using the piglet model. We tracked VH usage in DNA rearrangements and in VDJ transcripts throughout 75 days of gestation (DG) in outbred fetuses, thereafter in outbred germfree and colonized isolator piglets, isolator piglets infected with swine influenza and in conventionally reared nematode-infected adults. Seven VH genes account for >90% of the pre-immune repertoire which is the same among tissues and in both transcripts and DNA rearrangements. Statistical modeling supports the view that proportional usage of the major genes remains constant during fetal life and that postnatal usage ranking is similar to that during fetal life. Changes in usage ranking are developmental not antigen dependent. In this species exposure to environmental antigens results in diversification of the repertoire by somatic hypermutation of the same small number of VH genes that comprise the pre-immune repertoire, not by using other VH gene available in the germline. Therefore in swine a small number of VH genes shape the antibody repertoire throughout life questioning the need for extensive VH polygeny.

1. Introduction

The mammalian antibody repertoire is very large and with few exceptions, antibodies specific to all environmental and synthetic antigens and some autoantigens are recognized (Glanville et al., 2009; Weigert et al., 1970). The textbook paradigm is that such broad specificity rests on the large number of variable heavy and light chain gene segments in the repertoire that through somatic recombination adaptively generates a diverse spectrum of VDJ (heavy chain) and VJ (light chain) combinations (Cohn et al., 1980; Cohn and Langman, 1990) that become further diversified after antigen encounter by somatic hypermutation (SHM) and through the use of other VH, DH and JH recombinations. The pre-immune repertoire appears to have evolved to provide B cell receptors (BCR) of broad specificity and immediate protection that can later be somatically (adaptively) diversified to provide more specific and effective protection.

Among the germline-encoded variable gene segments, those in the variable heavy chain locus (VH) play the major role in the specificity of the binding site (Padlan, 1994). Furthermore, the diversity generated by recombination to yield CDR3 seems to dwarf the importance of combinatorial VH and VL diversity. This was previously suggested from our statistical studies (Butler et al., 2000a) and empirically demonstrated by Xu and Davis (2000) using a transgenic mouse with only one functional VH gene but an intact DH and JH genome. Such mice were capable of making antibodies to a wide array of antigens although restricted in response to polysaccharides. These observations question the paradigm that repertoire diversification is combinatorially dependent and leave unexplained the reason for the extensive VH polygeny reported in

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doi:10.1016/j.molimm.2011.09.018

eutherian mammals. Original studies estimated there were ~1000 VH genes in mice (Kabat et al., 1991; Schroeder, 2006) hundreds in humans and ~500 in rabbits that are grouped into clans and families (Kirkham et al., 1992). Those in humans are comprised of members of 7 families, in mice 15 families while in swine and rabbit, all belong to the ancestral VH3 family (Schroeder et al., 1990). In the last decade genome studies have allowed the number of VH genes in mice, humans and rabbits to be revised down to ~100 in humans and mice (Lefranc, 2003; Riblet, 2004) and ~100–200 in rabbits (Mage et al., 2006). However, species variation exists; less than 30 VH genes have been reported for swine and cattle (Butler, 2006). In both humans and swine only 40–70% of these are functional (Eguchi-Ogawa et al., 2010; Matsuda et al., 1993). By contrast, bats have ≈236 members in the VH3 family alone and in addition, four other families homologous to VH1, VH4, VH5 and VH7 in humans for which the number of family members is unknown (Bratsch et al., 2011).

Within these germline VH repertoires, usage is biased. In fetal humans and during B cell development in mice, initial studies reported that usage of VH genes near the 3′ end of the locus was preferred (Schroeder et al., 1987; Yancopoulos et al., 1984). This was reinforced by data showing that newborn rabbits use their 3′ gene ~90% of the time (Knight, 1992). However, complete physical mapping of the human VH locus does not support this association between location and usage (Matsuda et al., 1993) and we share the same observation for swine (Eguchi-Ogawa et al., 2010). In humans there is disproportionate representation of V3–23, V3–30, V3–33 and V4–34 (Glas et al., 2000). Similar preferences have been reported in mice (Gu et al., 1991; Sheehan et al., 1993).

In all of these studies, we noticed there had been no systematic vertical studies on VH usage spanning the period from the earliest onset of fetal B cell lymphogenesis to sexual maturity that included neonates maintained germfree or exposed to normal gut flora or infections as well as older conventionally reared animals. Our earlier report of restricted usage of VH genes in fetal piglets was based on a limited sampling, did not distinguish usage before and after transcription, was reported when the VH loci had not been mapped, and did not follow VH usage into postnatal life (Sun and Butler, 1996). Therefore we chose to advance our knowledge on this subject by monitoring VH usage in fetal germfree piglets colonized and virus-infected isolator piglets and conventionally reared young pigs that were parasite-infected. All animals were extensively outbred and originated from farms in four different states.

Since placentation in swine, and probably all artiodactyls (Brambell, 1970) prevents maternal Ig and other proteins from crossing the placenta, maternal regulation of fetal B cell development *in utero* can be eliminated as a cause of changes in fetal VH usage (Butler, 1974; Butler and Kehrle, 2005). This sets artiodactyls apart from mice, humans and rabbits in which IgG transferred across the placenta has been shown to effect immune development (Rodkey and Adler, 1983; Wang and Shlomchik, 1998; Wikler et al., 1980; Yamaguchi et al., 1983). Furthermore and in contrast to mice, humans and rabbits, swine produce precocial offspring that can be recovered by caesarian surgery and then reared in isolator facilities in which contact with environmental influences or maternal immune products are controlled by the experimenter (Butler and Sinkora, 2007; Butler et al., 2009a; Kim et al., 1968; Tlaskalova-Hogenova et al., 1994). These features, considered in context with the small number of VH genes, all of one family, two DH and one JH gene segment that are used to create the pre-immune repertoire (Butler et al., 1996; Eguchi-Ogawa et al., 2010; Sun and Butler, 1996), provide a manageable experimental setting for vertical studies on VH usage during fetal and postnatal life; a single primer set recovers all VDJ rearrangements in this species. This comparatively simple organization of the porcine genome has also provided the molecular basis for the generation of B cell deficient pigs for eventual transgenic use in production of humanized antibodies (Mendicino et al., 2011; Ramsoondar et al., 2011). Furthermore, swine have long been used in medical science (Tumbleson and Schook, 1996) including in xenotransplantation (Sachs et al., 2001) and more recently as a model for cystic fibrosis (Abu-El-Haigi et al., in press; Rodgers et al., 2008). Thus, the use of swine/piglets models in biomedical research seemed to warrant a better understanding of how their antibody repertoire develops in this species.

Table 1
Treatment groups, number of animals and number of clones examined.

Group Studied	Number of animals	Number of clones	
		DNA	cDNA
20 DG Yolk Sac	18	809	
30 DG Fetal Liver	4	584	
50 DG Fetal Liver	4	320	513
95 DG B.M.	5	672	411
95 DG Spleen	5	408	647
95 DG IPP	5	806	413
5-week Germ free	4		654
5-week Colonized/virus	4		625
5-week PIC	11		1444

Our results indicate that VH usage remains highly restricted in fetal piglets, that the same small number of VH genes that comprise the pre-immune repertoire also dominate the repertoire postnatally regardless of age or environmental antigen exposure and that postnatal repertoire diversification results from SHM of this same small number of VH genes.

2. Material and methods

2.1. Fetal tissues

Pregnant gilts were euthanized at 20 DG, 30 DG, 50 DG and 95 DG; yolk sac was recovered at 20 DG, fetal liver at 30 DG and 50 DG and bone marrow, spleen and ileal Peyers patches (IPP) at 95 DG. It has been previously shown that VDJ rearrangement occurs first in yolk sac, and then proceeds to the fetal liver at DG 30 and the bone marrow at DG 60 (Sinkora et al., 2003). Up to five fetuses per gilt were recovered (Table 1).

2.2. Isolator piglets

Pregnant gilts at 112 DG were anesthetized, their fetuses recovered by Caesarian aseptic procedures and then transferred to germfree isolators as previously described (Butler et al., 2008; Lemke et al., 2004). In isolators they were reared on sterile formula and: (a) maintained germfree, (b) colonized with a defined probiotic exclusion culture comprised of 10species that was developed at the USDA Regional Laboratory, College Station, TX (Harvey et al., 2005) or (c) infected with swine influenza (S-FLU. The S-FLU isolate used was the A/Swine/Iowa/15/1930, and H1N1 subtype that has been used extensively as a laboratory strain (Vincent et al., 2006). For challenge, pigs were physically restrained and given a 1 ml dose (1×10^4 $CCID_{50}$) intranasally of S-FLU that had been propagated in MDCK cells. For the purpose of this study, isolator piglets in the bacterial colonized and influenza infected groups were treated as one group (Col/S-FLU). Piglets were distributed (four/isolator) so that any one isolator contained animals derived from different mothers to avoid genetic bias. All piglets were regularly monitored using fecal and skin swabs for bacteria and their general health scored according to an established system. Weekly blood samples were obtained for measurement of Ig levels, for CBCs analysis and PBMC recovered for potential analysis of VH gene usage as was routinely done in studies of this type (Butler et al., 2000b; Lemke et al., 2004; Vincent et al., 2006).

2.3. Parasite-infected conventional piglets (PIC)

Since it is known that intestinal parasitism substantially influences adaptive immunity (Dawson et al., 2005), 4½ to 5-month old conventionally raised pigs from Maryland were inoculated with either 20,000 infectious *Ascaris suum* or 30,000 infectious *Trichuris suis* eggs and euthanized 11 and 21 days after inoculation, respectively. These times represent phases in the infection when parasitic larvae have migrated from the liver, lungs and small intestine (*A. suum*) or continue to develop in the caecum and proximal colon (*T. suis*). Both mesenteric lymph nodes (MLN) and tracheal–bronchial lymph nodes (TBLN) were recovered and stored in liquid nitrogen.

2.4. Origin of animals and approval for animal studies

Animals used were from various breeds and their crosses that included Yorkshires, Landrace and Durocs obtained form certified suppliers in South Dakota, Iowa, Wisconsin and Maryland. Fetal and isolator piglets from Iowa and South Dakota were derived from pregnant gilts purchased from a herd that was free of porcine reproductive and respiratory syndrome virus and swine influenza virus. Presumably they would be, or may have been colonized with common swine pathogens, but the infections were subclinical. Through husbandry practices (including commercially available vaccine against porcine parovirus and several sevovars of Leptiosira) the owner has maintained a sow herd that has no recognized clinical disease. PICs were derived from boars from a four-way crossbred composite BX line (Duroc X maternal Landrace X terminal Landrace X Yorkshire) designed by scientists at the USDA ARS US Meat Animal Research Center, Clay Center, NE to be genetically similar to genetics in the commercial swine industry at the time they were born; the genetics of the gilts are predominantly of the BX composite line. Animal were from a herd screened yearly for PRRSV, influenza (H1N1 and H3N2), pseudorabies, and brucellosis by the Veterinary Services Group at the Beltsville Agricultural Research Center and has been negative for these infections. New breeding boars that have been brought into the herd were pre-screened and then quarantined for 30 days before use. All animal studies were approved by the animal use committees at the National Animal Disease Laboratory and the Diet, Genomics and Immunology Laboratory both of the USDA and the Department of Veterinary Science of South Dakota State University. Further details concerning the experimental procedures used regarding the animals studied can be found in previous publications (Butler et al., 2002, 2005, 2009b, in press-a, 2011; Dawson et al., 2005; Mateo et al., 2011).

2.5. Tissue from isolator piglets

At 6 weeks, which is 5 weeks post viral inoculation or colonization with normal gut flora, treated and controls were euthanized and a variety of lymphoid tissues collected and stored in liquid nitrogen for molecular biological studies. The bone marrow (BM), IPP and spleen were the tissues of greatest interest in fetal and germfree controls while the MLN and IPP are most likely to be affected in colonized and parasite-infected animals and TBLN in those infected with S-FLU. BM was recovered from long bones after removal of cartilaginous ends, extrusion with saline and preservation in TriZol or DNA Zol (Invitrogen, Carlsbad, CA).

2.6. Preparation of DNA and cDNA

Tissues that had been stored in liquid nitrogen were pulverized and DNA extracted into DNAZol as previously described (Sun et al., 1998a). RNA was extracted from the same tissues and treated with DNase for two rounds for 30 min at 37 °C and then converted to cDNA (Butler et al., 2008; Sun and Butler, 1996; Sun et al., 1998a). VDJ rearrangements from DNA and cDNA were recovered using a hemi-nested PCR using 5′ FR1 and anti-sense JH followed by 3′ FR1 and anti-sense JH in the second round (Butler et al., 2006). The products were tested for size on an agarose gels, and were then excised from the gel, cloned into pCR4 TOPO, and amplified in Top 10 *Escherichia coli* cells.

2.7. VH gene nomenclature and sequence

The vernacular and the international Im-MunoGeneTics database (IMGT) designations for porcine VH gene are used; the latter are based on mapping the most 3′ VH genes in the locus (Eguchi-Ogawa et al., 2010). VH genes in the IMGT system are designated by increasing numbers from 3′ to 5′. VHC and VHZ have not been mapped and therefore have no IMGT designation. There are two VHA and two VHB genes in the genome. We designated them as recently duplicated VH genes because their CDR sequences were almost identical with differences confined to changes in FR3. The more upstream VHA (IGHV10) is designated as VHA* and requires a special probe for identification (Suppl. Table I) that binds in the FR3 region for identification. VHB (IGHV6) and VHB* (IGHV12) cannot be reliably distinguished by hybridization. Thus, usage of VHB and VHB* is reported as one gene (Figs. 1, 2 and 4).

2.8. Determination of VH usage by hybridization

Transformed top 10 bacterial cells (see above) were cloned and those harboring plasmids with inserts were transferred to 96-well plates and grown for 18 h. Bacterial cells were then lysed and plasmid DNA recovered and transferred to nylon membrane (Butler et al., 2006; Sun et al., 1998a). Plasmid DNAs transferred to the membranes were then sequentially hybridized with VH gene specific probes for sequences in CDR1 and CDR2 of the major genes (Butler et al., 2006; Suppl. Table I). The specificity and stringency requirement used have been previously described (Sun et al., 1998a,b). Briefly, stringency was adjusted so that no cross-hybridization occurred. Clones that failed to hybridize with ^{32}P probes for both CDR1 and CDR2 sequences but hybridized with a pan-specific probe that binds to FR2 (Suppl. Table I) were designated as unknown (UNK) clones or "non-hybridizing" VH clones. These include major VH genes that were somatically mutated and seldom-used VH genes (Butler et al., 2006). Those that initially hybridized with one CDR probe but not the other were separately enumerated (see RDI equation below). This hybridization method gave highly reproducible results when the same sample was repeatedly analyzed (Sun et al., 1998b). Most recently we re-addressed this by sequencing 75 hybridizing clones and 65 UNK clones. The rate of misidentification of hybridizing clones was 2.6% while 7.5% of UNK clones should have been detected by hybridization, Since hybridization is correct 90–95% of the time, we have confidence that this error rate does not alter the major findings obtained with the >8000 clones used in this study.

Since sequential hybridization of the VH-containing clones on membranes with up to eleven probes is labor intensive, we developed a cocktail system, which was substituted in most postnatal studies. One cocktail was comprised of CDR1 gene-specific probes and the second by CDR2 specific-probes (Suppl. Table I). We compared the performance of this cocktail method with sequential hybridization using individual gene-specific probes (Suppl. Figure 1).

2.9. Repertoire diversification index (RDI)

The RDI employed is a modification of that previously published (Butler et al., 2006) that was improved having available a larger

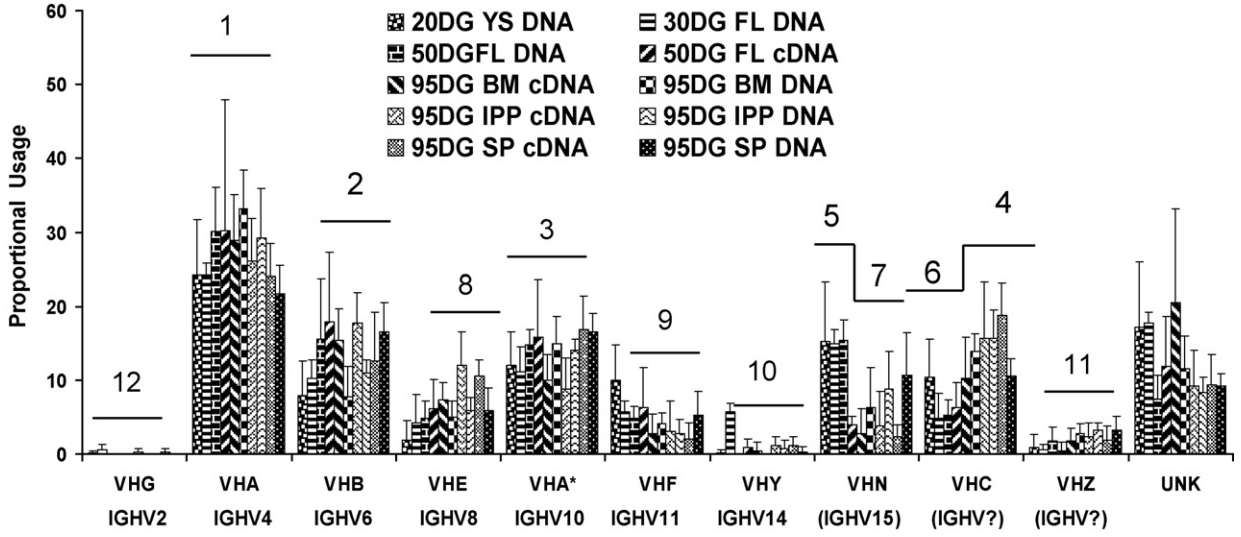

Fig. 1. Proportional VH usage throughout fetal development. More than 5500 VDJ clones recovered from DNA and cDNA from various lymphoid tissues from DG20 to DG95 fetuses were tested by sequential hybridization with 11 VH gene specific probes (Suppl. Table I). The number of animals and clones for each sample tested are summarized in Table 1. BM = bone marrow; IPP = ileal Peyers patches; FL = fetal liver; Sp = spleen; YS = yolk sac. The VH genes are designated by both their vernacular name and their IMGT designations (if assigned). The VH genes are displayed on the *x*-axis according to their 3′ to 5′ order in the locus. VHN and VHZZ are currently considered alleles. Values given are the mean and standard deviations (error bars) among the animals for each sample type tested. Horizontal bars indicate the rank of VH usage with rank numbers indicated above. Statistical analyses indicate that these horizontal lines indicate that usage in constant throughout fetal development with exception of VHN and VHC in which usage is age-related.

data base. Developing an RDI using CDR gene-specific ^{32}P probes (Suppl. Table I) is based on several empirical observations. First, seven VH genes account for >90% of the pre-immune repertoire (Fig. 1). Second, the frequency of clones designated as unknown in fetal animals is ~15% (Fig. 1) but increases with environmental antigen exposure. Their identity must be determined by sequence analysis (see below) although this information is not needed in the calculation of the RDI. Third some VH gene clones hybridize with

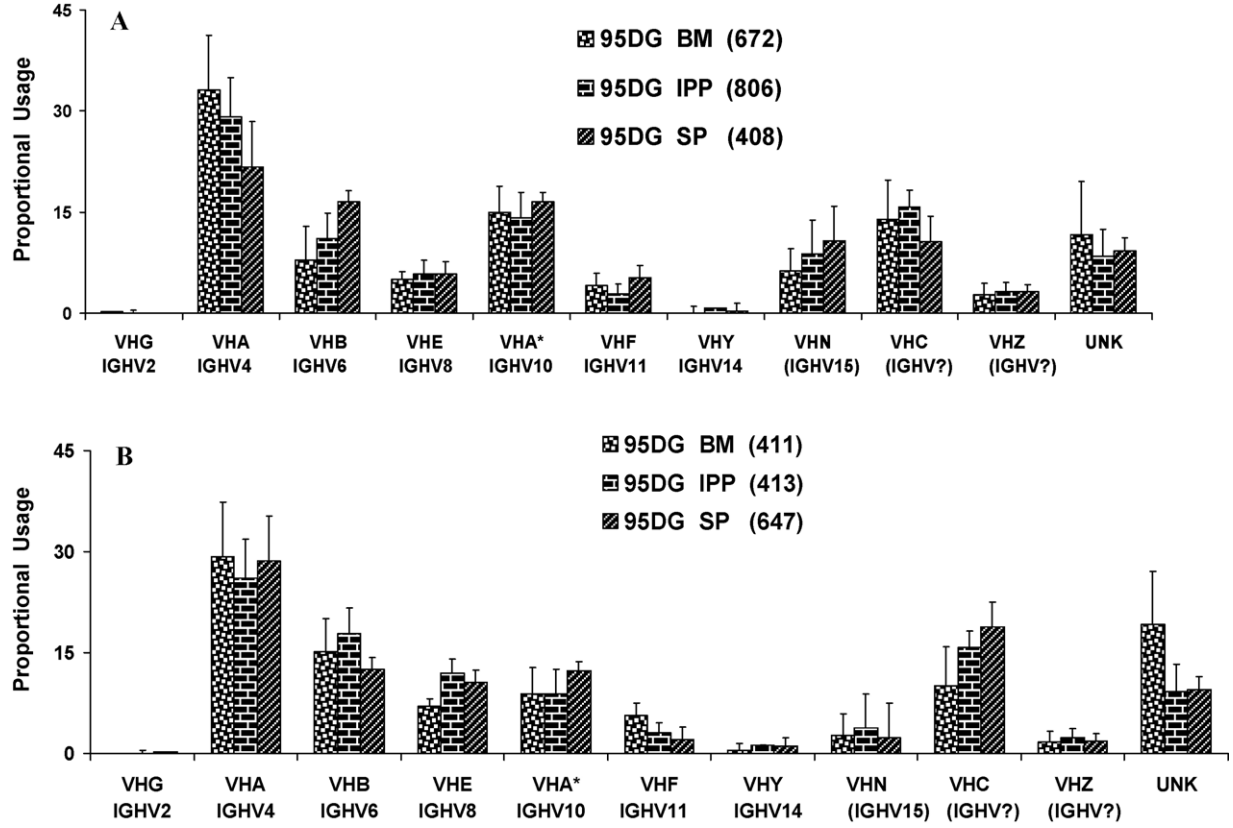

Fig. 2. Comparison of VH usage in DNA versus cDNA from 95 DG spleen, IPP and BM. (A) Data from DNA; (B) Data from cDNA from the same animals. The procedures used and the legend are the same as for Fig. 1. The number of piglets and DNA and cDNA VH clones are given in Table 1 and shown in parenthesis on the figure.

Table 2
Summary of non-hybridizion V_H genes.

Age/tissue	Proportion of UNK genes	Proportion of V_H clones	
		Major	Rare
20 DG/yolk sac	10%	65(13)	35(7)
30 DG/liver	14%	50(5)	50(5)
50 DG/liver	12%	82(9)	18(2)
95 DG/Sp	10%	55(6)	45(5)
95 DG/B.M.	19%	50(7)	50(7)
95 DG/IPP	10%	50(7)	50(7)
5-wk GF[a]	34%	73(8)	27(3)
5-wk C/V[b]	88%	85(51)	15(9)
5-wk PIC[c]	95%	72(28)	28(11)

[a] Tissues examined were IPP and MLN.
[b] Tissues examined were Sp and MLN.
[c] Tissues examined were BLN and MLN.

one CDR probe (or probe cocktail) but not with the other and this is incorporated into the RDI equation.

$$\mathrm{RDI} = \frac{\mathrm{UNK}^a + [\mathrm{CDR1} + \mathrm{CDR2}]^b/2}{(\mathrm{VHA},\ \mathrm{VHA}*,\ \mathrm{VHB},\ \mathrm{VHB}*\mathrm{VHC},\ \mathrm{VHE},\ \mathrm{VHF})^c}$$

a: UNK are non-hybridizing VH gene clones that could represent mutated VH genes or the use of seldom-used VH genes for which CDR-specific probes have not been developed.
b: Clones hybridizing with only one CDR-specific probe, i.e. CDR1 or CDR2.
c: These are referred to as "major VH genes" (see Fig. 1;see Suppl. Table I).

2.10. Identification of non-hybridizing (UNK) VDJ clones

VDJ clones that failed to hybridize with CDR-specific probes for the major VH genes (Suppl. Table I) i.e. those designated as UNK or "non-hybridizing", were propagated overnight, harvested, purified, restriction digested with EcoRI and evaluated on agarose gels to confirm the insert size (Table 2). All appropriate-sized inserts were sequenced in the DNA Core Facility of the University of Iowa using the four-color ABI Prism DNA Analyzer (Applied Biosystems). Data presented are based on 110 UNK clones from fetal piglets, 11 from germfree isolator piglets, 60 from colonized and S-FLU-infected isolator piglets and 39 from conventionally reared parasitized pigs (PIC).

2.11. Determination of somatic hypermutation frequency

The parent genes for UNK clones and hybridizing VH gene clones were identified by sequence comparison to the ~30 reported porcine VH genes which probably include some allotypic variants (Butler et al., 2006). Identification was based on the minimum number of changes needed for the UNK sequence to agree with an established sequence, including pyrimidine bias. Mutations in each gene segment were normalized to the length of each segment and nucleotides changes were expressed per kilobase or as protein changes per 330 amino acids.

$$\mathrm{Mutation\ \ frequency} = \frac{\mathrm{mutations\ \ observed}}{\mathrm{segment\ \ length}} \times 1000$$
$$(\mathrm{or}\ \ \times 330\ \ \mathrm{for\ \ VH\ \ protein})$$

2.12. Determination of serum Ig levels

The concentration of IgM, IgG and IgA in serum was measured by sandwich ELISA as previously described (Butler et al., 2000a,b, 2005).

2.13. Statistical analysis and modeling

VH usage for each fetal piglet at each time point was compiled in Excel spreadsheets. The patterns of usage by VH genes from >5500 VDJ clones representing 10 different VH genes and the UNK category from 10 different sources were examined for each gene separately, and within and between the 4 different time points; (DG 20, 30, 50, 95) according to the method described by the R. Development Core Team (Bates and Maechler, 2009). Five different models were compared using the AIC criterion (Akaike, 1983) to select models that best fit the data. An additional analysis examined the pattern of usage of each gene within each source by transforming the percentage of each of the 11 identified genes to a rank for each source and animal. The ranks were analyzed with the linear model that used fixed effects for genes and sources (R. Development Core Team). The model selection procedure was similar to that described above.

In all other situations mean difference was examined using the Student's *t*-test and ANOVA programs provided in the stat package of the Prism graphics program.

3. Results

3.1. VH usage in VDJ rearrangements and mRNA during fetal life

Consistent with earlier studies (Sinkora et al., 2003) VDJ rearrangements were detected in yolk sac at DG 20, next in fetal liver at 30 DG and then in BM at 65 DG. Tdt, RAG-1 and signal joint circles can be recovered at these sites and B cell lymphogenesis continues thereafter in BM after birth (Sinkora et al., 2003; Butler et al., unpublished). Fig. 1 is based on >5500 clones and shows that seven major VH genes (VHA, VHB, VHA*, VHN, VHE, VHF and VHC) account for ~93% of the total repertoire throughout fetal development and four account for ~73% extending the more limited data that we have previously reported (Butler et al., 2006; Sun and Butler, 1996; Sun et al., 1998a). Usage is not dependent on position in the genome since VHG (IGHV2), which is the most 3′ functional VH gene, is seldom used while VHN (IGHV15) can account for up to 13% of the repertoire in early fetuses (Eguchi-Ogawa et al., 2010). Inspection shows a surprisingly invariant usage profile for sites sampled from 20–95 DG. The obvious exceptions are the decreased usage of VHN (IGHV15) and the reciprocal increase usage in VHC at DG 95. The order of usage of the 10 VH genes and UNK is illustrated by the horizontal bars in Fig. 1. VHA (IGHV4) alone accounts for 25% of total VH usage and together with its duplicate (IGHV10) accounts for more than one third of the repertoire.

3.2. Modeling supports a pattern of nearly constant usage of major VH genes in fetal life

Studies show that the model in which proportional, i.e. rank order usage is constant at each of the 4 time points is the model that best fits the data. Accepting this model also indicates that the variability between sources at the same time point is small. This best model shows that for VHA, VHA*, VHF, VHC and VHZ there is no difference in the population percentages for any source at any at any time point. However, differences in VHE, VHN, VHC and VHZ usage that are obvious by inspection (Fig. 1) were confirmed by modeling. Specifically three of the four genes (VHE, VHC and VHZ) display higher proportional usage at DG 95 than at earlier time points while this is reversed for VHN with a clear preference at DG 20 compared to DG 95 ($p < 10^{-3}$).

3.3. VH usages in rearrangements and transcripts have similar profiles in primary and secondary lymphoid tissues

Spleen and IPP both appear to be secondary B cell lymphoid tissues in swine (Butler et al., 2011). VH usage at these sites is the same as in BM, regarded as a primary lymphoid tissue for B cells (Fig. 2A). The usage profile for VH genes in VDJ rearrangements in DNA (Fig. 2A) was compared with the profile obtained using transcripts from spleen, BM and IPP from the same piglets at DG 95 (Fig. 2B) by using the same modeling program described in Section 3.2 above. Only small differences were seen (Fig. 2B). VHA, VHB and VHC played major roles as transcripts and VHY and VHZ minor roles. Notably VHA* was less-represented in transcripts from very early fetuses. Despite this deviation, VH usage in VDJ transcripts is generally representative of VH usage in DNA rearrangements in both primary and secondary lymphoid tissues. We separately analyzed differences in usage among tissues. Only two of 30 comparison differed in cDNA ($p < 0.03$) and three in DNA ($p < 0.03$). These differences all involved spleen.

3.4. The RDI and the proportion of UNK gene clones increases after exposure to PAMP-bearing antigens

When exposed to colonizing bacteria in isolator units or during conventional-rearing, piglets respond with a general Th1 response. This exposure also promotes development of an adaptive immune response (Butler et al., 2002, 2005). In contrast, when piglets are deliberately inoculated with nematode parasites (PIC treatment group), they produce a strong TH2 response (Dawson et al., 2005; Urban et al., 1991). In addition to these PIC piglets, and those colonized in isolators, we include isolator piglets maintained germfree (GF) in which their only environmental exposure is food antigens, and isolator infected with S-FLU which promotes a rapid antibody response that clears the virus in 7–10 days and the lung lesions in <21 days (Vincent et al., 2006).

Fig. 3 shows that RDI values increase in parallel to the increase in the proportion of UNK VH gene clones (data not shown) since the proportion of UNK is a major ingredient in the RDI equation (see Section 2). As defined, the designation UNK includes all VH gene clones that hybridize with the conserved FR2 region probe but fail to hybridize with probes for the CDR1 and CDR2 regions of the major VH genes used (Suppl Table I). Thus, UNK VH genes could be somatic mutants of these major VH genes or represent seldom-used VH genes among the <30 VH genes described for swine (Butler et al., 2006). Table 2 shows that the proportion of UNK genes is 2-fold higher in 5 weeks GF piglets than in fetal piglets and reaches ~90% in PIC and colonized/S-FLU -infected piglets. This is ~10-fold higher than in fetal piglets. The number of non-hybridizing major VH genes accounts for ~75% of VH usage in antigenized groups and GF animals (Table 2) while usage of major and minor VH genes among UNK VH gene clones is nearly equal in fetal piglets

3.5. The frequency of SHM primarily depends on environmental exposure

Young piglets exposed to environmental stimuli, such as within isolators like those colonized with a defined flora or infected with S-FLU or conventionally reared and nematode infected, show a significantly elevated mutation frequency compared to fetal piglets and those maintained GF in isolators (Fig. 4). The frequency of mutation in GF piglets was indistinguishable from that seen in fetal piglets. Thus, repertoire diversification by SHM is not age-dependent but rather depends on encounter with PAMP-bearing microorganisms.

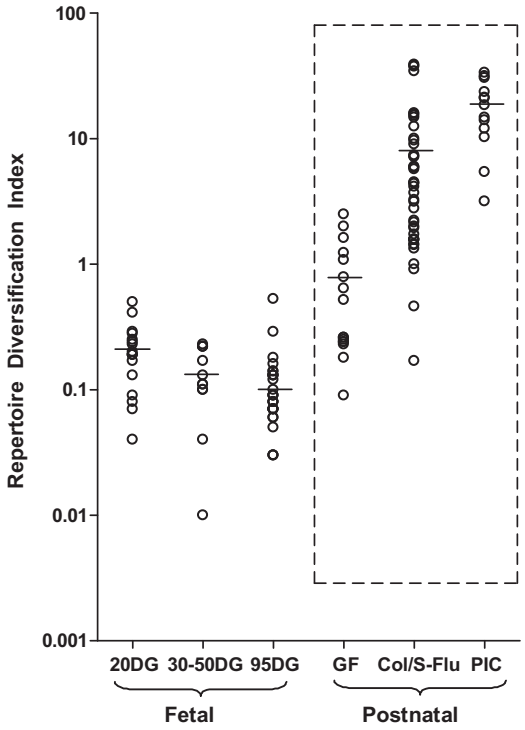

Fig. 3. Repertoire diversification index (RDI) for three groups of fetal piglets and three groups of postnatal piglets compared using a log scale. DG = day of gestation; GF = germfree; C/V = colonized and S-FLU-infected; PIC = parasite-infected conventional piglets. The number of animals and clones tested are given in Table 2. The horizontal bar in the scattergram represents the mean RDI for each group. The area bracketed further identifies data from postnatal piglets. A two-sided ANOVA indicated that the RDI for GF isolator piglets was significantly higher than in fetal piglets (*P*, 0.005) and that Col/S-FLU and PIC pigs had a significantly higher RDI than GF isolator piglets (*P*, 0.01 and <0.001, respectively).

3.6. SHMs preferentially accumulate in CDR1 and CDR2

Tables 3 and 4 compare the frequency of SHM in the ~950 bp segment that comprises hybridizing and non-hybridizing (UNK) VH gene clones at the nucleotide (Table 3) and deduced amino acid level (Table 4). Mutation frequency is given for the framework (FR) and CDR regions of the genes. The overall mutation frequency was highest in non-hybridizing VH genes clones (32.4 ± 25.5 per kilobase versus 5.8 ± 6.6; Table 3) and 19.5 ± 15.4 per 330 amino acids versus 2.4 ± 5.4 (Table 4). The frequency of mutation among seldom-used VH genes (minor) was the same as for hybridizing VH genes. Table 3 shows that the frequency of SHM in CDR regions of hybridizing VH gene clones is 200-fold lower than in non-hybridizing VH genes ($p < 0.001$) whereas differences in SHM in FR regions of these two categories of VH genes is not significant ($p = 0.1$–0.8 depending on age). This shows that SHM accumulate in the CDR regions of non-hybridizing genes. Table 3 also shows that SHM occurs in both CDR and FR region of VH genes recovered from the earliest rearrangements in 20 DG yolk sac. Thus some level of SHM occurs in the absence of environmental antigen and in fact appears lower in CDR regions than in FR regions at this time ($p < 0.01$–0.0001 depending on age). Not surprisingly, hybridizing VH genes show almost no mutations in CDR1 and CDR2, thus supporting the distinction between hybridizing and non-hybridizing VH genes that is part of the RDI equation employed to study repertoire diversification. Data also show that mutations in FR2 are low or absent, supporting the use of a FR2-specifiic probe to identify all VDJ-containing clones. While there was considerable variation in SHM, variation was less at the protein level (Table 4) due to codon redundancy.

Table 3
Frequency and distribution of SHM of rearranged hybridizing and non-hybridizing VH genes.

Source		NR		Region of gene					
				FR1	CDR1	FR2	CDR2	FR3	Total
20 DG	Hybrid	31	Avg	1.0	0.0	0.0	0.0	6.1	3.90
			S.D.	5.6	na	na	na	10.0	5.65
	Non-hybrid	13	Avg	9.6	102.6	14.7	91.6	4.3	20.9
			S.D.	19.7	84.4	20.7	73.9	5.2	13.1
	Rare	8	Avg	6.9	33.3	0.0	5.3	7.9	7.5
			S.D.	13.8	50.4	na	15.9	6.9	5.1
30–50 DG	Hybrid	15	Avg	8.3	0.0	1.6	0.0	14.8	8.52
			S.D.	14.3	na	6.1	0.0	8.4	6.35
	Non-hybrid	12	Avg	0.0	55.6	6.0	36.6	9.3	13.4
			S.D.	na	47.8	14.8	64.4	14.3	15.8
	Rare	8	Avg	3.9	0.0	0.0	0.0	6.0	3.7
			S.D.	11.0	na	na	na	5.6	3.5
95 DG	Hybrid	43	Avg	4.0	0.0	0.0	0.0	17.1	10.26
			S.D.	12.8	na	na	na	10.7	6.06
	Non-hybrid	17	Avg	7.2	41.0	3.7	44.4	9.2	13.4
			S.D.	13.7	58.0	8.9	64.7	7.8	6.0
	Rare	22	Avg	3.9	0.0	0.0	3.1	12.2	7.4
			S.D.	14.0	na	na	10.5	9.9	4.5
5-week Germ free	Hybrid	65	Avg	2.9	0.0	1.5	0.0	4.4	2.2
			S.D.	10.7	0.0	5.8	na	6.4	3.0
	Non-hybrid	20	Avg	9.4	53.3	6.0	48.1	10.7	16.0
			S.D.	23.4	51.0	17.5	66.9	12.2	15.2
	Rare	20	Avg	0.0	13.3	1.2	1.9	7.1	5.1
			S.D.	0.0	59.6	5.3	8.3	8.1	7.6
5-week Col/SIV	Hybrid	16	Avg	2.0	0.0	1.5	3.0	13.4	6.9
			S.D.	7.8	na	6.0	11.9	14.1	7.2
	Non-hybrid	59	Avg	16.4	111.9	7.7	100.6	25.2	33.3
			S.D.	30.4	128.9	12.8	89.1	20.6	21.6
	Rare	15	Avg	16.7	31.1	4.8	57.5	16.9	19.5
			S.D.	26.1	49.5	9.9	81.3	12.3	14.7
PIC	Hybrid	7	Avg	8.9	0.0	3.4	0.0	15.9	7.9
			S.D.	15.2	na	9.0	na	16.5	8.6
	Non-hybrid	41	Avg	35.8	172.4	10.5	210.9	29.6	52.7
			S.D.	51.6	145.3	14.1	136.0	20.3	30.5
	Rare	1	na	31.3	0.0	0.0	0.0	7.9	8.5
			S.D.	na	na	na	na	na	na
All	Hybrid	177	Avg	3.4	0.0	1.0	0.3	9.8	5.8
			S.D.	10.9	na	4.7	3.6	11.0	6.6
	Non-hybrid	162	Avg	18.0	109.3	8.3	112.4	20.2	32.4
			S.D.	33.3	117.7	13.6	111.9	18.6	25.5
	Rare	74	Avg	5.8	13.3	1.3	13.4	10.8	9.2
			S.D.	15.9	42.4	5.4	42.4	9.8	9.7

3.7. Serum Ig levels parallel the increase in the frequency of UNK, SHM and the RDI

Table 5 summarizes the data on Ig levels of the major isotypes for animals in the various treatment groups. These data reveal a non-significant increase in serum IgG and IgA levels in 5-week-old germfree isolator piglets compared to newborns, but a real increase in IgM. Piglets exposed to PAMP-bearing microorganism and their antigens in isolator had levels of IgM, IgG and IgA that were elevated 7-fold above those in GF isolator piglets, while parasite-infected animals that were conventionally reared had IgM and IgA levels elevated >600-fold and IgA levels elevated 90-fold above those of GF piglets. These results provide supporting evidence that the adaptive immune response system was activated by environmental antigens.

3.8. The order of major VH usage in UNK VH gene clones resembles the pattern seen in the pre-immune repertoire

Sequence analysis of 171 UNK VH clones revealed that ~75% were mutated versions of the major porcine VH genes that comprise the pre-immune repertoire (Table 2). The rank order of usage of these UNK VH genes among piglets maintained germfree or exposed to various environmental antigens (antigenized) is given in Table 6. The identity of these genes was determined by sequence analysis and their rank usage order compared to usage among hybridizing VH genes from early (20–50 DG) and late (95 DG) fetuses.

Table 6 shows that VHA is the most frequently used gene in all four categories and that VHG, VHZ and VHY are consistently the least-used. VHB, VHE and VHF maintain a middle ranking (5–8) in all categories. Table 5 also suggests that there are clear age-dependent patterns for some VH genes; VHC usage ranks second and third in older animals but is seldom used by early fetuses. VHA* usage decreases after birth, regardless whether in GF or antigenized young pigs and VHN appears to be an early VH gene, although its usage is not position-dependent in the porcine VH genome. Accepting these age-dependent changes in VH usage, young animals exposed to antigen diversify their repertoire by SHM of the same VH genes that comprise the pre-immune repertoire (Table 6; Fig. 1).

Table 4
Frequency and distribution of SHM in deduced structure of hybridizing and non-hybridizing VH genes.

Source		NR		Region of gene					
				FR1	CDR1	FR2	CDR2	FR3	Total
20 DG	Hybrid	31	Avg	0.0	0.0	0.0	0.8	0.0	2.3
			S.D.	na	na	na	4.3	na	3.7
	Non-hybrid	13	Avg	3.3	11.1	66.6	15.9	83.3	13.9
			S.D.	4.9	16.4	56.8	25.5	50.2	18.2
	Rare	8	Avg	0.8	2.6	30.7	1.8	8.5	6.1
			S.D.	2.8	9.2	44.0	6.6	16.2	7.3
30–50 DG	Hybrid	15	Avg	0.0	0.0	9.5	1.7	20.4	8.5
			S.D.	na	na	24.2	6.4	76.3	13.0
	Non-hybrid	12	Avg	0.0	0.0	38.9	2.0	30.8	13.2
			S.D.	na	na	34.3	6.9	38.1	11.4
	Rare	8	Avg	0.0	0.0	0.0	0.0	0.0	0.0
			S.D.	na	na	na	na	na	na
95 DG	Hybrid	43	Avg	0.0	0.0	0.0	0.0	0.0	1.2
			S.D.	na	na	na	na	na	3.3
	Non-hybrid	17	Avg	1.3	5.1	35.9	0.0	29.3	7.9
			S.D.	3.5	12.5	69.9	0.0	45.7	10.7
	Rare	22	Avg	0.4	1.4	0.0	0.0	1.5	0.3
			S.D.	2.0	6.8	na	na	7.6	1.6
5-week germ free	Hybrid	65	Avg	2.8	0.0	1.5	0.0	1.5	1.6
			S.D.	10.3	na	5.8	na	3.1	2.4
	Non-hybrid	20	Avg	3.0	36.6	3.6	30.1	4.4	8.6
			S.D.	9.3	45.7	11.6	42.1	6.5	8.0
	Rare	20	Avg	0.0	0.0	1.2	0.0	2.8	2.1
			S.D.	na	na	5.3	na	3.9	4.0
5-week Col/SIV	Hybrid	16	Avg	1.9	0.0	1.5	2.3	7.4	5.3
			S.D.	7.6	na	5.9	9.3	6.8	4.2
	Non-hybrid	59	Avg	6.2	62.1	2.4	61.8	12.4	17.5
			S.D.	12.3	79.1	7.3	53.5	12.2	12.9
	Rare	15	Avg	6.5	14.3	3.4	20.4	8.5	8.7
			S.D.	12.5	37.3	8.4	35.4	9.4	6.5
PIC	Hybrid	7	Avg	8.6	0.0	3.4	0.0	5.7	6.0
			S.D.	14.8	na	9.0	na	6.0	7.6
	Non-hybrid	41	Avg	16.2	99.1	4.6	116.8	19.0	30.1
			S.D.	28.0	79.0	9.5	70.9	14.3	17.7
	Rare	1	na	0.0	0.0	0.0	0.0	0.0	0.0
			S.D.	na	na	na	na	na	na
All	Hybrid	177	Avg	1.6	0.8	1.1	1.9	2.8	2.4
			S.D.	7.5	7.1	5.0	21.8	5.7	5.4
	Non-hybrid	162	Avg	8.2	64.9	3.9	68.7	12.9	19.5
			S.D.	18.1	73.5	11.1	63.9	13.2	15.4
	Rare	74	Avg	1.9	7.4	1.2	5.9	3.4	3.6
			S.D.	7.6	25.8	5.2	18.5	6.3	5.9

4. Discussion

The essential vertical studies designed to follow VH usage through various stages of fetal development and beyond into postnatal life in mice or humans in a controlled manner are missing from the literature. Performing such studies in mice and humans would be challenging since a very large number of VH-JH primers sets (30–50) might be needed and it is difficult to imagine how such data could be quantified given the inherent bias among primer sets. The use of mice and humans is further complicated by *in utero* transfer of regulatory factors such as maternal IgG (Rodkey and Adler, 1983; Wang and Shlomchik, 1998; Wikler et al., 1980; Yamaguchi et al., 1983) to say nothing about the difficulty of controlling the postnatal environment of infants and the altricial offspring of mice (Zemlin et al., 2010). While high through-put sequencing of VDJ rearrangements has allowed for the identification of many VH gene polymorphisms, it has not addressed the ontogenetic aspect (Weinstein et al., 2009; Boyd et al., 2010). To overcome these difficulties, omissions and potential ambiguities, we chose to focus

Table 5
Serum immunoglobulin concentration (μg/ml) in piglets from various groups used in the study.

	NR[a]	IgM	IgG	IgA
Fetal (90-101DG)	38	1.15 ± 2.04	6.8 ± 6.1	0.4 ± 0.44
Newborn	16	0.83 ± 0.55	29.3 ± 18.1	2.8 ± 1.2
5-week GF	8	7.32 ± 2.35	40.97 ± 6.33	2.62 ± 0.53
5-week Col/SIV	16	64.67 ± 20.49	313.52 ± 58.7	16.80 ± 2.86
5-month PIC	9	5177 ± 1138	25,780 ± 6044	231 ± 76

[a] NR: number of animals tested. Data given as mean ± S.D.

Table 6
The rank order of usage among major VH genes identified by sequence analysis from UNK cloness.[a]

Treatment	VHG	VHA	VHB	VHE	VHA*	VHF	VHY	VHN	VHC	VHZ
20–50 DG	10	1	3	7	2	6	8	4	5	9
95 DG	10	1	3	7	2	6	9	5	4	8
UNK-A	10	1	5	3	7	6	8	4	2	9
UNK-GF	10	1	4	6	7	8	5	2	3	9

[a] Frequency of usage is indicated by rank; 1 = most frequent, 10 = least frequent. It should be noted that 12 ranks were indicated in Fig. 1 because the rank of V_HN and V_HC changes with age. Rank is given for hybridizing early fetal (20–50 DG) and late fetal (95 DG) and for V_H genes identified by sequencing UNK V_H clones from piglets maintained germfree (GF) and antigenized (A) piglets.

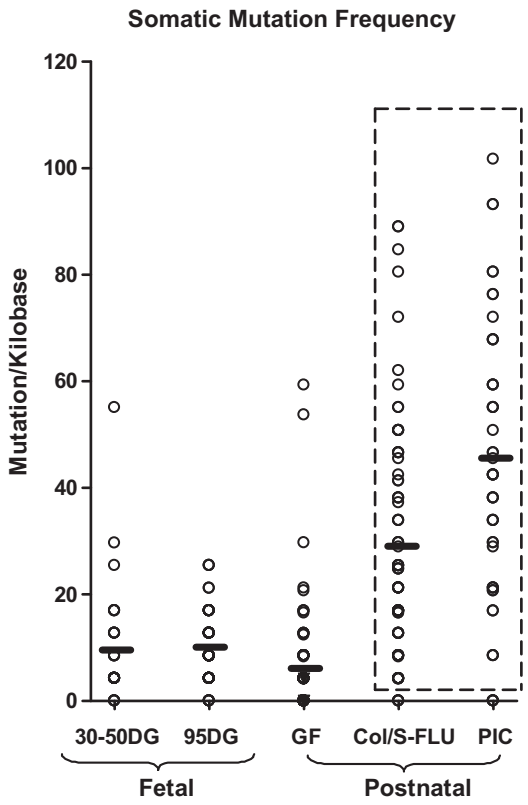

Fig. 4. Frequency of somatic hypermutation (SHM). Scattergram shows the distribution of sequence with mutations while the horizontal bars indicate the mean. Antigenized piglets (Col/S-FLU and PIC) are bracketed. ANOVA shows that antigenized piglets have a significantly higher frequency that those not exposed to environmental antigen ($P < 0.001$).

on the piglet model. Swine have no transfer of passive immunity *in utero*, their offspring are precosial and have a manageable germline repertoire comprised of <30 VH3 genes, including a single JH so that a single PCR primer set can recover all VDJ rearrangements without bias in swine.

Our observations indicate that VH usage is non-random and proportional usage fits a model showing that fetal VH usage remains constant with some changes that are developmental but not antigen dependent. It should not be without notice that data are from outbred animals that are a mixture of breeds from several states and therefore different founder animals. Therefore they are not strain-biased as in studies with lab rodents. This constancy is especially surprising since the animals were outbred and individual variation might be expected to mask a species pattern.

Studies over the years reveal that original estimates of the number of germline VH genes in the mouse and human genome were overestimated and that extensive germline diversity is not universal among mammals (Butler, 1997). For example, swine and domesticated ruminants probably have <30 VH genes and these belong to a single family (Berens et al., 1997; Saini et al., 1997; Sinclair et al., 1997; Sun et al., 1994). The fact that these species use only a few of these genes and the rabbit primarily one gene to encode their pre-immune repertoire (Knight, 1992) raises the question of the need and function of large numbers of VH genes for host protection. The extensive germline repertoire of VH genes in mammals was originally considered the basis of their ability to recognize nearly any self, foreign or environmental antigen; this repertoire increased the chance that the host can successfully make an immune response to any threatening pathogen (Cohn et al., 1980; Cohn and Langman, 1990; Langman and Cohn, 1987; Rajewsky et al., 1987). This concept continues to be the one presented in immunology textbooks (Abbas and Lichtman, 2001; DeFranco et al., 2007; Janeway et al., 2005; Kuby, 2007; Roitt et al., 2001). While this may be true in Zebrafish that use 50–86% of their repertoire (Weinstein et al., 2009) or perhaps some bats (Bratsch et al., 2011) studies by Xu and Davis (2000) have shown that so long as the DH and JH regions remain intact, a mouse with a single VH gene could respond to nearly all antigens. The exception is responses to bacterial polysaccharides and these responses may depend on "natural antibodies" (Ochsenbein and Zinkernagel, 2000) encoded by VH genes other than the one used in the transgenic mouse. The origin of the germline diversity of VH genes regardless of value, presumably the result of gene duplication and genomic gene conversion similar to what has been proposed for the origin of MHC polygeny (Marchalonis et al., 1996) and IgG subclass diversification in mammals (Butler et al., 2009b, in press-a). Obviously the extent of this process must have differed among major species. Duplication and conversion events were apparently restricted to the VH3 family in swine and rabbit, and the VH4 family in cattle. In contrast the VH gene repertoire in mice expanded to 15 families and in humans to seven families (Honjo and Matsuda, 1995) and to at least five families in bats (Bratsch et al., 2011). In any case, duplicated VH genes retain the canonical structures in their CDR regions, presumably a necessity for forming or stabilizing an effective binding site (Chothia et al., 1989). However, there appears to be no general pattern relating the number of VH genes or VH gene families to the ability to respond efficiently to antigenic stimulation.

Preferential VH usage is based on studies in mice (Yancopoulos et al., 1984) and human (Schroeder et al., 1987) that indicated that during fetal development, preferentially used VH genes were clustered at the 3′ end of the VH locus suggesting an ontogenetic position effect. This is consistent with the observation that <90% of the pre-immune repertoire in rabbits is comprised of the most 3′ VH gene (Knight, 1992). However, these observations in humans were not sustained during studies on VH gene mapping and usage (Matsuda et al., 1993) and cannot explain the pattern seen in swine in which the 3′-most functional VH gene VHG (IGHV2) is seldom used while VHN (IGHV15) can comprise 15% of the pre-immune repertoire (Eguchi-Ogawa et al., 2010; Fig. 1).

Data presented are the result of characterizing VH usage through 75 days of gestation (DG) and postnatally in isolator piglets maintained either germfree or infected with S-FLU or colonized with a defined gut flora. Data from these groups were compared to those from conventionally reared, sexually mature pigs exposed to nematode infections. Our studies show that seven VH genes comprise >90% of the pre-immune repertoire and that the same genes comprise >80% of the postnatal repertoire albeit having undergone SHM (Table 2). As shown in mice (Berek and Milstein, 1987) mutations accumulate in CDR1 and CDR2 (Tables 3 and 4). Presumably the mutations also accumulate in CDR3 but are difficult to quantify because of nucleotide additions and exonuclease activity acting on DH and JH segments (Butler et al., 2000a). Since CDR1, CDR2 and CDR3 encode sequences that coalesce to form the antibody binding site; SHM in these segments is no doubt the major source of binding site diversity (Padlan, 1994). Altogether the observation that only a few VH genes comprise the repertoire and their usage fits a computer model of constant VH usage. Nevertheless, certain VH genes show an age-dependent pattern. For example, the tendency for increased usage of VHC in older fetuses and postnatally (regardless of treatment) versus the reciprocal decrease in VHN usage in older fetuses and postnatally in antigenized piglets is noteworthy (Fig. 1 and Table 6). This seems to suggest an age-dependent rather than an antigen-dependent change suggesting a programmed expression for "early" and "late" VH genes.

Our data also show that the frequency of SHM (Tables 3 and 4), the proportion of VH genes clones that failed to hybridize with gene-specific CDR1 and CDR2 probes, i.e. UNK clones (Table 2) and

the RDI (Fig. 3) are positively correlated with an increase in serum Ig levels of the three major isotypes (Table 6). Thus, diversification at the molecular level also translates to increased Ig synthesis and secretion. Consistent with our previous studies, the present data show that adherent bacteria or exposure to purified PAMPs is required for these adaptive changes in repertoire and Ig production (Butler et al., 2002, 2005). We show here that germfree piglets exposed only to dietary antigen (no PAMPs) show no increase in SHM (Fig. 4) in serum IgG and IgA, the major Igs that characterize adaptive immune responses (Table 6). The increase in IgM in GF animals presumably reflects age-related proliferation of naïve B cells but with antigen-dependent switch to IgG and IgA. These findings generally agree with those of Lanning et al. (2000) in rabbits, except we observed an increase in the RDI compared with fetal piglets (Fig. 5). The modest increase in total IgM in GF piglets may reflect polyclonal B cell activation poorly up-regulates AID resulting in little SHM and CSR.

The constancy of VH usage may not be unique to swine. Unfortunately, comparable vertical studies in mice and human could not be identified. Should this phenomenon also occur in mice, rabbits and human that has so many germline VH genes, the evolutionary significance of VH polygeny would remain unexplained. Perhaps early vertebrates and primitive mammals originally used a system that depended on VH polygeny for antibody diversity. We know that in sharks combinatorial diversity was absent or limited because V–D–J elements are fused in the genome (Dooley and Flajnik, 2006). Furthermore, elasmobranchs, teleosts and amphibians lack GCs where SHM and CSR occur following antigen stimulation (Du Pasquier et al., 1998). In these early vertebrates, somatic events appear to have been less important and that VH polygeny was more important. Thus early vertebrates and some mammals may depend more on combinatorial diversity and less on somatic recombination and SHM while the opposite is true for swine and other artiodactyls that have a relatively small number of VH genes (Berens et al., 1997; Butler, 2006; Saini et al., 1997; Sinclair et al., 1997). The little brown bat has >250 VH3 genes plus probably >100 additional VH genes that belong to at least four other families, all of which have human homologs (Bratsch et al., 2011). The same study shows that SHM is rare in this bat suggesting that antibody diversity in this bat may rely more heavily on selection from a large pool of germline VH genes (Cohn et al., 1980; Rajewsky et al., 1987), which may be the case in Zebrafish that lack GC (Weinstein et al., 2009). Thus, a highly diverse VH repertoire does not appear to be universally needed by all mammals for host survival. It may, however, be necessary for primitive mammals like bats and early vertebrates in which GC are absent or poorly developed.

Regardless of difference in VH polygeny among mammals, selective VH usage is still unexplained. At least for swine the "position concept" initially promoted from studies in mice, humans and rabbit, does not occur in swine (Eguchi-Ogawa et al., 2010; Fig. 1). Perhaps emphasis should focus on other structural aspects of the locus. Since all swine VH genes have identical leader sequences and recombination signal sequences (RSSs) intronic sequences or the critical distances between preferentially used VH genes and the heavy chain promoter may be involved. In mice, 5′ VH genes are seldom used (Sheehan et al., 1993) so that accessibility for RAG and other important factors involved in V(D)J recombination may be most important. The idea that the pre-immune repertoire has been selected for "useful" specificities (Ratcliffe, 2006) such as for VH genes that encode the specificity of natural antibodies that recognize bacterial polysaccharides or other "danger antigens" (Matzinger, 1994) is plausible. It was noted by Xu and Davis (2000) that this property was lacking in transgenic mice with only one VH gene that make adaptive responses to most antigens but appear to lack responses to bacterial polysaccharides. A phenomenon termed "Deceptive Imprinting" (Kohler et al., 1994; Tobin et al., 2008) showed that responses made early upon exposure to pathogens occur without immune maturation processes such as SHM or CSR, consistent with the broad specificity of BCRs of the pre-immune repertoire and the concept of natural antibodies (Ochsenbein and Zinkernagel, 2000).

In conclusion, the vertical studies presented indicate that the random use of VH genes described in textbooks that is based on mouse studies is not applicable to the piglet model. Rather, our findings confirm the non-random VH usage reported for species like rabbit, indicating that usage is intrinsically programmed. However, our results take this concept a step further by showing that even after antigen exposure, this programmed usage is maintained, i.e. the same selected few are diversified by SHM as opposed to using other VH genes available in the germline.

Acknowledgements

Research supported by a contract from Biological Mimetics, by NSF grant IOS 0077237 and grant 07-210 from the National Porkboard.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molimm.2011.09.018.

References

Abbas, A.K., Lichtman, A.H., 2001. Immunobiology 5: The Immune System in Health and Disease, 5th ed. Garland Publishing.
Akaike, H., 1983. Information measures and model selection. Bull. Int. Stat. Inst. 44, 277–291.
Abu-El-Haigi, M., Sinkora, M., Meyerholz, D.K., Welsch, M.J., McCray, P.B.,Jr, Butler, J.E., Uc, A. An activated immune and inflammatory response targets the newborn pig cystic fibrosis pancreas. Pancreatology, in press.
Bates, D., Maechler, M., 2009. lme4: Linear Mixed-Effects Models Using S4 Classes. R Foundation for Statistical Computing, Vienna, Austria, ISBN 3-900051-07-0, http://www.R-project.org.
Berek, C., Milstein, C., 1987. Mutation drift and repertoire shift in the maturation of the immune response. Immunol. Rev. 96, 23–41.
Berens, S.J., Wylie, D.E., Lopez, O.J., 1997. Use of a single V-H family and long CDR3s in the variable region of cattle Ig heavy chains. Int. Immunol. 9, 189–199.
Boyd, S.D., Gaeta, B.A., Jackson, K.J., Fire, A.Z., Marshall, E.L., Merker, J.D., Maniar, J.M., Zhang, L.N., Sahaf, B., Jones, C.D., Simen, B.B., Hanczaruk, B., Nguyen, K.D., Hadeau, K.C., Egholm, M., Mikos, D.B., Zehnder, J.L., Collins, A.M., 2010. Individual variation in the germline Ig gene repertoire inferred from variable region gene rearrangements. J. Immunol. 184, 6986–6992.
Brambell, F.W.R., 1970. The Transmission of Passive Immunity from Mother to Young. North-Holland. Publ. Co., Amsterdam.
Bratsch, S., Wertz, N., Chaloner, K., Kunz, T.H., Butler, J.E., 2011. The little brown bat displays a highly diverse VH, DH and JH repertoire but little evidence of somatic hypermutation. Dev. Comp. Immunol. 35, 421–430.
Butler, J.E., 1974. Immunoglobulins of the mammary secretions. In: Larson, B.L., Smith, V. (Eds.), Lactation, a Comprehensive Treatise, vol. III. Academic Press, New York, pp. 217–255 (chapter V).
Butler, J.E., Sun, J., Navarro, P., 1996. The swine immunoglobulin heavy chain locus has a single J_H and no identifiable IgD. Int. Immunol. 8, 1897–1904.
Butler, J.E., 1997. Immunoglobulin gene organization and the mechanism of repertoire development. Scand. J. Immunol. 45, 455–462.
Butler, J.E., Sun, J., Weber, P., Francis, D., 2000b. Antibody repertoire development in fetal and neonatal piglets. III. Colonization of the gastrointestinal tracts results in preferential diversification of the pre-immune mucosal B-cell repertoire. Immunology (British) 100, 119–130.
Butler, J.E., Weber, P., Sinkora, M., Sun, J., Ford, S.J., Christenson, R., 2000a. Antibody repertoire development in fetal and neonatal piglets. II. Characterization of heavy chain CDR3 diversity in the developing fetus. J. Immunol. 165, 6999–7011.
Butler, J.E., Weber, P., Sinkora, M., Baker, D., Schoenherr, A., Mayer, B., Francis, D., 2002. Antibody repertoire development in fetal and neonatal piglets. VIII. Colonization is required for newborn piglets to make serum antibodies to T-dependent and type 2 T-independent antigens. J. Immunol. 169, 6822–6830.
Butler, J.E., Kehrle Jr., M.E., 2005. Immunocytes and immunoglobulins in milk. In: Mestecky, J., Lamm, M.E., Strober, W., McGhee, J.R., Mayer, L., Bienenstock, J. (Eds.), Mucosal Immunology. , 3rd ed. Academic Press, NY, pp. 1763–1793.
Butler, J.E., Francis, D., Freeling, J., Weber, P., Sun, J., Krieg, A.M., 2005. Antibody repertoire development in fetal and neonatal piglets. IX. Three PAMPs act

synergistically to allow germfree piglets to respond to TI-2 and TD antigens. J. Immunol. 175, 6772–6785.
Butler, J.E., 2006. Preface: why I agreed to do this. In: Butler, J.E. (Guest Ed.), Antibody Repertoire Development. Developmental and Comparative Immunology 30, 1–17.
Butler, J.E., Weber, P., Wertz, N., 2006. Antibody repertoire development in fetal and neonatal pigs. XIII. "Hybrid VH genes" and the pre-immune repertoire revisited. J. Immunol. 177, 5459–5470.
Butler, J.E., Sinkora, M., 2007. The isolator piglet: a model for studying the development of adaptive immunity. Immunol. Res. 39, 33–51.
Butler, J.E., Weber, P., Wertz, N., Lager, K.M., 2008. Porcine reproductive and respiratory syndrome virus (PRRSV) subverts development of adaptive immunity by proliferation of germline-encoded B cells with hydrophobic HCDR3s. J. Immunol. 180, 2347–2356.
Butler, J.E., Lager, K.M., Splichal, I., Francis, D., Kacskovics, I., Sinkora, M., Wertz, N., Sun, J., Zhao, Y., Brown, W.R., DeWald, R., Dierks, S., Muyldermanns, S., Lunney, J.K., McCray, P.B., Rogers, C.S., Welsh, M.J., Navarro, P., Klobasa, F., Habe, F., Ramsoondar, J., 2009a. The Piglet as a model for B cell and immune system development. Vet. Immunol. Immunopathol. 128, 147–170.
Butler, J.E., Wertz, N., Deschacht, N., Kacskovics, I., 2009b. Porcine IgG: structure, genetics and evolution. Immunogenetics 61, 209–230.
Butler, J.E., Sun, X.-Z., Wertz, N. Immunoglobulin polygeny: an evolutionary perspective. In: Zorbic P. (Ed.), Gene Duplication/Book 2, InTech gen access publ. ISBN 978-953-308-57-8, in press-a.
Butler, J.E., Mateo, K., Sun, X.-Z., Wertz, N., Sinkora, M., Harvey, R., Francis, D.L., 2011. Antibody repertoire development in fetal and neonatal piglets XX. The ileal Peyers patches are not a site of B cell lymphogenesis and are not required for systemic B cell proliferation and Ig synthesis. J. Immunol., 187, in press-b.
Chothia, C., Lesk, A.M., Tramontano, A., Levitt, M., Smith-Gill, S.J., Air, G., Sheriff, S., Padlan, E.A., Davies, D., Tulip, W.R., Colman, P., Spinelli, S., Alzaro, P., Poljak, R., 1989. Conformations of immunoglobulin hypervariable regions. Nature 342, 877–883.
Cohn, M., Langman, R.E., 1990. The protecton: the unit of humoral immunity selected by evolution. Immunol. Rev. 115, 11–147.
Cohn, M., Langman, R.E., Geckeler, W., 1980. Diversity in immunology. In: Fougereau, M., Dausset, J. (Eds.), Progress in Immunology, 4th Intl Congr Immunol. Acad. Press, N.Y. 153–201.
Dawson, H.D., Beshah, E., Nishii, S., Solano-Aquilar, G., Morimoto, M., Zhao, A., Madden, K.B., Ledbetter, T.K., Dubey, J.P., Shea-Donohue, T., Lunney, J.K., Urban Jr., J.F., 2005. Localized multi-gene expression patterns support an evolving Th1/Th2 paradigm in response to infections with *Toxoplasma gondii* and *Ascaris suum*. Infect. Immun. 73, 1116–1128.
DeFranco, A.L., Locksley, R.M., Robertson, M., 2007. Immunity. Sinauer Associates, Inc., Sunderland, MA, 387 pp.
Dooley, H., Flajnik, M.J., 2006. Antibody repertoire development in cartilagenous fish. Dev. Comp. Immunol. 30, 43–56.
Du Pasquier, L., Wilson, M., Greenberg, A.S., Flajnik, M.F., 1998. Somatic mutation in ectothermic vertebrates: musings on selection and origins. Curr. Top. Microbiol. Immunol. 229, 199–216.
Eguchi-Ogawa, T., Sun, X.-Z., Wertz, N., Uenishi, H., Puimi, F., Chardon, P., Wells, K., Tobin, G.J., Butler, J.E., 2010. Antibody repertoire development in fetal and neonatal piglets. XI. The relationship of VDJ usage and the genomic organization of the variable heavy chain locus. J. Immunol. 184, 3734–3742.
Glanville, J., Zhai, W., Telma, D., Huerta, G., Mehta, G.R., Ni, I., Mei, L., Saundar, P.D., Day, G.M.R., 2009. Precise determinations of the diversity of combinatorial libraries give insight in the human immunoglobulin repertoire. Proc. Natl. Acad. Sci. U.S.A. 106, 20216–20221.
Glas, A.M., van Monfort, E.H.N., Milner, E.C.B., 2000. The human antibody repertoire: old notions, current realities and VH gene-dependent biases. In: Zanetti, M., Capra, J.D. (Eds.), The Antibodies, vol. 6. Harwood Academic Publishers, Amsterdam, pp. 63–79.
Gu, H., Tarlinton, D., Muller, W., Rajewsky, K., Forster, I., 1991. Most peripheral B cells in mice are ligand restricted. J. Exp. Med. 173, 1357–1371.
Harvey, R.B., Anderson, R.C., Genovese, K.J., Callaway, T.R., Nisbet, D.J., 2005. Use of competitive exclusion to control enterotoxigenic strains of *Escherichia coli* in weaned pigs. J. Anim. Sci. 83, E44–E47.
Honjo, T., Matsuda, F., 1995. Immunoglobulin heavy chain loci of mouse and humans. In: Honjo, T., Alt, F.W. (Eds.), Immunoglobulin Genes. Academic Press, N.Y., p. 443.
Janeway, C.A., Travers, P., Walport, M., Schlomchik, M.J., 2005. Immunobiology. Garland Press, New York, 823 pp.
Kabat, E.A., Wu, T.T., Perry, H.M., Gottesman, K.S., Foeller, C., 1991. Sequences of Proteins of Immunological Interest, 5th ed. NIH Publications, Washington, D.C.
Kim, Y.-B., Bradley, S.G., Watson, D.W., 1968. Ontogeny of the immune response. V. Further characterization of the 19S and 7 S IgG immunoglobulins in the true primary responses in germfree colostrum-deprived piglets. J. Immunol. 101, 224–236.
Kirkham, P.M., Mortari, F., Newton, J.A., Schroeder, H.W., 1992. Immunoglobulin VH clan and family identity predicts domain structure and many influence antigen binding. EMBO J. 11, 603–609.
Knight, K.L., 1992. Restricted VH usage and generation of antibody diversity in rabbit. Ann. Rev. Immunol. 10, 593–616.
Kohler, H., Muller, S., Nara, P.L., 1994. Deceptive imprinting in the immune response against HIV-1. Immunol. Today 10, 475–478.
Kuby, J., 2007. In: Kindt, T., Goldsby, R., Osborne, B. (Eds.), Immunology. W.H. Freeman, New York.
Langman, R.E., Cohn, M., 1987. The E–T (elephant–tadpole) paradox necessitates the concept of a unit of B cell function: the protecton. Mol. Immunol. 24, 675–697.
Lanning, D., Sethupathis, P., Rhee, K.J., Zhai, S.K., Knight, K.L., 2000. Intestinal microflora and diversification of the rabbit antibody repertoire. J. Immunol. 165, 2012–2019.
Lefranc, M.-P., 2003. IMGT. The international ImMunoGeneTics database. Nucleic Acid Res. 31, 307–310.
Lemke, C.D., Haynes, J.S., Spaete, R., Adolphson, D., Vorwald, A., Lager, K., Butler, J.E., 2004. Lymphoid hyperplasia resulting in immune dysregulation is caused by PRRSV infection in pigs. J. Immunol. 172, 1916–1925.
Mateo, K.S., Ayres, J., Zhao, M., Butler, J.E., Francis, D.H., 2011. Intestinal resection and anastomosis in neonatal gnotobiotic piglets. J. Am. Assoc. Lab. Anim. Sci. 50, 361–364.
Mage, R.G., Lanning, D., Knight, K.L., 2006. B cell and antibody repertoire development in rabbits: the requirement of gut-associated lymphoid tissues. Dev. Comp. Immunol. 30, 137–153.
Marchalonis, J.J., Schluter, S.F., Bernstein, R.M., Shanxiang, S., Edmundson, A.B., 1996. Phylogenetic emergence and molecular evolution of the immunoglobulin family. Adv. Immunol. 70, 417–506.
Matsuda, F., Shin, E.K., Nagaoka, H., Matsumura, R., Haino, M., Fukita, Y., Takaishi, S., Imai, T., Riley, J.H., Amand, R., Soeda, E., Honjo, T., 1993. Structure and physical map of 64 variable segments in 3′ 0 8-megabase region of the human immunoglobulin heavy-chain locus. Nat. Genet. 3, 88–94.
Matzinger, P., 1994. Tolerance, danger and the extended family. Ann. Rev. Immunol. 12, 991–1045.
Mendicino, M., Ramsoondar, J., Phelps, C., Vaught, T., Ball, S., Dai, Y., ReRoith, T., Monahan, J., Chen, S., Dandro, A., Boone, J., Jobst, P., Vance, A., Wertz, N., Berman, Z., Sun, X.-Z., Polejaeva, I., Butler, J.E., Ayares, D., Wells, K., 2011. Targeted disruption of the porcine immunoglobulin heavy chain locus produces a null phenotype. Transgenic Res. 20, 625–641.
Ochsenbein, A.F., Zinkernagel, R., 2000. Natural antibodies and complement link innate and acquired immunity. Immunol. Today 1, 624–630 (package version 0.999375-35) http://CRAN.R-project.org/package=Ime4.
Padlan, E.A., 1994. Anatomy of the antibody molecule. Mol. Immunol. 31, 169–217.
R Development Core Team. R: a language and environment for statistical computing.
Ratcliffe, M.J.H., 2006. Antibodies, immunoglobulin genes and the bursa of Fabricius in chicken B cell development. Dev. Comp. Immunol. 30, 101–118.
Rajewsky, K., Forster, I., Cumano, A., 1987. Evolutionary and somatic selection of the antibody repertoire in the mouse. Science 238, 1088–1094.
Ramsoondar, J., Mendicino, M., Phelps, C., Vaught, T., Ball, S., Monahan, J., Chen, S., Dandro, A., Boone, J., Jobst, P., Vance, A., Wertz, N., Polejaeva, I., Butler, J.E., Dai, Y., Ayares, D., Wells, K., 2011. Targeted disruption of the porcine immunoglobulin kappa light chain locus. Transgenic Res. 20, 643–653.
Riblet, R., 2004. Immunoglobulin heavy chain genes of mouse. In: Honjo, T., Alt, F.W. (Eds.), Molecular Biology of B cells. Elsevier, Amsterdam, pp. 19–26.
Rodgers, C.S., Stoltz, D.A., Meyerholz, D.K., Ostedgaards, L.S., Rohhlima, T., Taft, P.J., Rogan, M.P., Pezuzulo, A.A., Karp, P.H., Itani, O.A., Kabel, A.C., Wohford-Lenae, C.L., Davis, G.J., Hanfland, R.A., Smith, T.L., Samuel, M., Wax, D., Murphy, C.N., Rieke, A., Whiteworth, K., Uc, A., Starner, T.D.S., Brogden, K.A.K.A., Shilyansky, J., McCray, P.D., Zabner, J., Prather, R.S., Welsch, M.J., 2008. Disruption of the CFTR gene produces a model of cystic fibrosis in newborn pigs. Science 321, 1837–1841.
Rodkey, L.S., Adler, F.L., 1983. Regulation of natural anti- allotypic antibody responses by network induced auto-anti-idiotypic responsiveness of their offspring. J. Exp. Med. 152, 1024–1035.
Roitt, I., Brostoff, J., Male, D., 2001. Immunology, 6th ed. Mosby, London.
Sachs, D.H., Sykes, M., Robson, M., Cooper, M.J., 2001. Xenotansplanation. Adv. Immunol. 79, 129–223.
Saini, S.S., Hein, W.R., Kaushik, A., 1997. A single predominately expressed polymorphic immunoglobulin VH gene family, related to mammalian group, I, clan, II, is identified in cattle. Mol. Immunol. 34, 641–651.
Schroeder Jr., H.W., 2006. Similarity and divergence in the development and expression of the mouse and human antibody repertoires. Dev. Comp. Immunol. 30, 119–135.
Schroeder Jr., H.W., Hillson, H.L., Perlmutter, R.M., 1987. Early restriction of human antibody repertoire. Science 238, 791–793.
Schroeder Jr., H.W., Hillson, H.L., Perlmutter, R.M., 1990. Structure and evolution of mammalian VH families. Int. Immunol. 2, 41–45.
Sheehan, K.M., Mainville, C.V.A., Willert, S., Brodeur, P.H., 1993. The utilization of individual VH exons in the primary repertoire of adult BALB/c mice. J. Immunol. 151, 5363–5375.
Sinclair, M.C., Gilchrist, J., Aitken, R., 1997. Bovine IgG repertoire is dominated by a single diversified VH gene family. J. Immunol. 159, 3883–3889.
Sinkora, M., Sun, J., Sinkorova, J., Christenson, R.K., Ford, S.P., Butler, J.E., 2003. Antibody repertoire development in fetal and neonatal piglets. VI. B cell lymphogenesis occurs in multiple sites with differences in the frequency of in-frame rearrangements. J. Immunol. 170, 1781–1788.
Sun, J., Butler, J.E., 1996. Molecular characteristics of VDJ transcripts from a newborn piglet. Immunology (British) 88, 331–339.
Sun, J., Hayward, C., Shinde, R., Christenson, R., Ford, S.P., Butler, J.E., 1998a. Antibody repertoire development in fetal and neonatal piglets. I. Four V_H genes account for 80% of V_H usage during 84 days of fetal life. J. Immunol. 161, 5070–5078.
Sun, J., Kacskovics, I., Brown, W.R., Butler, J.E., 1994. Expressed swine V_H genes belong to a small V_H gene family homologous to human V_H III. J. Immunol. 153, 5618–5627.

Sun, J., Shey, M., Butler, J.E., 1998b. Determination of gene usage by differential polymerase chain reaction product hybridization. Anal. Biochem. 260, 71–79.
Tlaskalova-Hogenova, H., Mandel, L., Trebichavsky, I., Kovurau, F., Barot, R., Sterzl, J., 1994. Development of immune responses in early pig ontogeny. Vet. Immunol. Immunopathol. 43, 135–142.
Tobin, G.J., Trujillo, J.D., Bushnell, R.V., Lin, G., Chaudhuri, A.R., Long, J., Barrera, J., Pena, L., Grubman, M.J., Nara, P.L., 2008. Deceptive imprinting and immune refocusing in vaccine design. Vaccine 26, 6189–6199.
Tumbleson, M., Schook, L. (Eds.), 1996. Advances in Swine in Biomedical Research. Plenum Press, NY.
Urban Jr., J.F., Katona, I.M., Paul, W.E., Finkelman, F.D., 1991. Interleukin 4 is important in protective immunity to a gastrointestinal nematode infection in mice. Proc. Natl. Acad. Sci. U.S.A. 88, 5513–5517.
Vincent, A.L., Lager, K.M., Ma, W., Lekcharonensuk, P., Gramer, M.R., Loiacona, C., Richt, J.A., 2006. Evaluation of hemogluttinin subtype 1 swine influenza viruses from the United States. Vet. Microbiol. 118, 212–222.
Wang, H., Shlomchik, M.J., 1998. Maternal Ig mediates neonatal tolerance in rheumatoid factor transgenic mice but tolerance breaks down in adult mice. J. Immunol. 160, 2263–2271.
Weigert, M.G., Cesari, I., Yonkovitch, J.I., Cohn, M., 1970. Variability in light chain sequences of mouse antibodies. Nature 228, 1045–1047.
Weinstein, J.A., Jiang, N., White III, R.A., Fisher, D.S., Quake, S.R., 2009. High-throughput sequencing of the zebrafish antibody repertoire. Science 8, 807–810, doi:10.1126/science.1170020.
Wikler, M., Demeur, C., Dewasne, G., Urbain, J., 1980. Immunoregulatory role of maternal idiotypes. Ontogeny of immune networks. J. Exp. Med. 152, 1024–1035.
Xu, J.L., Davis, M.M., 2000. Diversity in the CDR3 region of VH is sufficient for most antibody specificities. Immunity 13, 37–45.
Yamaguchi, N., Shimizu, S., Hara, A., Saito, T., 1983. The effector maternal antigenic stimulation upon the active immune responsiveness of their offspring. Immunology 50, 229–238.
Yancopoulos, G.D., Desiderio, S.V., Paskind, M., Kearney, J.F., Baltimore, D.V., Alt, F.W., 1984. Preferential usage of the most JH proximal VH gene segments in pre-B cell lines. Nature 311, 717–733.
Zemlin, M., Hoersch, G., Zemlin, C., Pohl-Schickinger, A., Hummel, M., Berek, C., Maier, R.F., Bauer, K., 2010. Postnatal maturation of the immunoglobulin heavy chain IgG repertoire in human preterm neonates is slower than in term neonates. J. Immunol. 178, 1180–1188.